JVI Accepted Manuscript Posted Online 23 March 2016
J. Virol. doi:10.1128/JVI.00422-16

Bluetongue virus NS4 protein is an interferon antagonist and a determinant of virus virulence

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Running title: Modulation of the IFN response by BTV

Abstract: 234 words**; Importance:** 129 words**; Main text:** 5469

ABSTRACT

Bluetongue virus (BTV) is the causative agent of bluetongue, a major infectious disease of ruminants with serious consequences to both animal health and the economy. The clinical outcome of BTV infection is highly variable and dependent on a variety of factors related to both the virus and the host. In this study, we show that the BTV non-structural protein NS4 favours viral replication in sheep, the animal species most affected by bluetongue. In addition, NS4 confers a replication advantage to this virus in primary sheep endothelial cells and in IFN-competent immortalized cell lines. We determined that in cells infected with a NS4 deletion mutant (BTV8ΔNS4) there is an increased synthesis of type I interferon (IFN) compared to cells infected with wild type BTV-8. In addition, using RNAseq, we show that NS4 modulates the host IFN response and downregulates mRNA levels of type I IFN and interferon stimulated genes. Moreover, using reporter assays and protein synthesis assays, we show that NS4 downregulates the activity of a variety of promoters such as the cytomegalovirus immediate early promoter, the IFN-β promoter and a promoter containing interferon-stimulated response elements (ISRE). We also show that the NS4 inhibitory activity on gene expression is related to its nucleolar localization. Furthermore, NS4 does not affect mRNA splicing or cellular translation. The data obtained in this study strongly suggest that BTV NS4 is an IFN-antagonist and a key determinant of viral virulence.

IMPORTANCE

Bluetongue is one of the main infectious diseases of ruminants and is caused by bluetongue virus (BTV), an arthropod-borne virus transmitted from infected to susceptible animals by *Culicoides* biting midges. Bluetongue has a variable clinical outcome that can be related to both virus and host factors. It is therefore critical to understand the interplay between BTV

49 and the host immune responses. In this study we show that a non-structural protein of BTV
50 (NS4) is critical to counteract the innate immune response of the host. Infection of cells with
51 a BTV mutant lacking NS4 results in increased synthesis of IFN-β and upregulation of
52 interferon stimulated genes. In addition, we show that NS4 is a virulence factor for BTV by
53 favouring viral replication in sheep, the animal species most susceptible to bluetongue.
54

INTRODUCTION

Bluetongue virus (BTV) is the causative agent of bluetongue, a major infectious disease of ruminants with serious consequences to both animal health and the economy (1). Throughout the twentieth century, bluetongue has occurred almost exclusively in tropical and sub-tropical geographical areas (2). However, in the last two decades the geographical limits of the disease have expanded and BTV is now endemic in more temperate areas such as Southern and Central Europe (3).

BTV is an arbovirus transmitted by *Culicoides spp.* biting midges and belongs to the *Orbivirus* genus within the *Reoviridae* (4, 5). The BTV genome consists of 10 dsRNA genome segments encoding for seven structural and four, possibly five, non-structural proteins (6-9). The core particle, formed by VP3 and VP7, encapsidates the viral genome segments, each associated with a replicase complex comprising VP1, VP4 and VP6 (6, 10-12). VP2 and VP5 constitute the outer capsid of the BTV virion and are responsible for cell attachment and entry (13-15). VP2 is the most variable BTV protein and determines the virus serotype, of which there are 27 described to date (16-19).

The BTV non-structural proteins play fundamental roles in virus replication. NS1 forms tubules in the cytoplasm of BTV infected cells and favours viral protein synthesis (20-22). NS2 is the major component of viral inclusion bodies (23-25), while NS3/NS3A play a critical role in virus intracellular trafficking and egress (26, 27). NS3 has also been shown to downregulate transcription from the IFN-β promoter in reporter assays (28). A putative fifth non-structural protein may be expressed from a conserved small open reading frame in segment 10 (29).

NS4 is a small protein (77-79 amino acid residues) encoded by an open reading frame (ORF) in genome segment 9 overlapping the larger ORF encoding for VP6 and with nucleolar

localization. Although NS4 has been shown to confer a replication advantage to BTV in cells treated with interferon, it is dispensable for BTV replication in IFN incompetent cell lines and has no impact on pathogenicity in IFNAR$^{-/-}$ mice (8, 9).

The clinical outcome of BTV infection is highly variable and dependent upon a variety of viral, host and likely environmental factors (30-35). Understanding the interplay between BTV and the host immune response will be central to identifying the virus and host determinants of disease susceptibility.

In order to establish a successful infection, BTV must overcome both physical and innate immune barriers. One of the key innate immune mechanisms to fight viral infections is the IFN system (36). Mammalian cells possess pattern-recognition receptors that detect incoming pathogen signatures and induce the synthesis and secretion of IFN-β. Secreted IFN signals in both an autocrine and paracrine fashion, leading to the transcription of hundreds of IFN-stimulated genes (ISGs), some of which have a direct or indirect antiviral effect (37). BTV is known to induce an IFN response, both *in vitro* and *in vivo*, and consequently it must possess countermeasures that allow the virus to replicate in the face of this host response (8, 38-43). Evidence to date suggests that BTV NS3 and NS4 are the viral proteins most likely responsible for the disruption of the cellular innate immune response to BTV infection (8, 28).

In this study, we investigated the interaction of BTV and the IFN response of the host. We demonstrate that NS4 is an important virulence factor in sheep, a natural host of BTV infection, and acts as an interferon antagonist.

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MATERIALS AND METHODS

Cell cultures. Vero, BHK21 and BSR cells (a clone of BHK21, kindly provided by Karl K. Conzelmann) (44) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). CPT-Tert cells are sheep choroid plexus cells immortalized with the simian virus 40 (SV40) T antigen and human telomerase reverse transcriptase (hTERT) (45). CPT-Tert cells were grown in Iscove's modified Dulbecco's medium (IMDM), supplemented with 10% FBS. A549 cells are derived from a human lung adenocarcinoma and were grown in DMEM supplemented with 10% FBS. All cell lines were cultured at 37°C in a 5% CO_2 humidified atmosphere.

Primary ovine endothelial (ovEC) cells were obtained as previously described (46). ovEC were seeded in 12-well plates and maintained in a low oxygen incubator (37°C, 5% CO_2 and 3% O_2). In this study, ovEC were passaged once before being used and cultured at 37°C in a 5% CO_2 humidified atmosphere.

Viruses. Wild type BTV8 (BTV8wt) and a NS4 deletion mutant (BTV8ΔNS4) viruses used in this study were obtained by reverse genetics as described previously (8, 47). Virus stock titres were determined by standard plaque assays using CPT-Tert cells (48). The defective interfering-rich (DI-rich) preparations of Sendai virus (SeV, Cantell strain) was generated by sequentially passaging the virus at high MOI as previously described (49). Encephalomyocarditis virus (EMCV) was used in interferon protection assays (see below) and was prepared as previously described (50).

Virus replication curves. Replication curves were carried out in either ovEC or A549 cells. Cells were infected with the appropriate virus (MOI = 0.01) and supernatants were collected at 24, 48 and 72h post-infection (pi). Supernatants were subsequently titrated by endpoint

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dilution analysis on BSR cells using the method of Reed and Muench and expressed as log_{10} ($TCID_{50}$/ml) (51). Each experiment was performed independently in triplicate using at least two different stocks of each virus.

Ethical Statement. All animal experimental procedures carried out in this study were approved by the ethical committee of the Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise "G. Caporale" (Teramo, Italy) (protocol no. 11427/2012) and further approved by the Italian Ministry of Health (Ministero della Salute) in accordance with Council Directive 86/609/EEC of the European Union and the Italian D.lgs 116/92.

***In vivo* pathogenicity studies.** Experiments were carried out using 15 sheep (Italian mixed-breed) in an insect-proof isolation unit. Before inoculation, all animals were confirmed to have no antibodies against BTV using a blocking enzyme-linked immunosorbent assay (ELISA) as previously described (52). The absence of BTV genome in blood samples of each animal was also confirmed by qRT-PCR as already described (30). Animals (n=5 per group) were infected with 5 ml of either BTV8wt or BTV8ΔNS4 (2×10^6 PFU in total) by multiple intradermal inoculations in the inner leg and prescapular areas. Negative controls were inoculated with 5 ml of mock-infected cell supernatants. Body temperature was recorded daily, beginning a week before inoculation, until day 14 pi and subsequently at days 17, 21, and 28. Fever was defined as rectal temperature above 40°C. EDTA blood samples were collected daily from all animals for 14 days pi and thereafter at days 17, 21, and 28, when the experiment was terminated. Blood samples were analyzed for the presence of viremia by qRT-PCR as previously described (30). Sera were collected from each animal on the day of the inoculation (day 0) and then at days 7, 14, 21, and 28 pi. The presence of neutralizing antibodies in infected sheep against BTV8 was assessed by virus neutralization assay as previously described (53).

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148 **Plasmids and antisera.** The open reading frame encoding NS4 was either amplified by PCR
149 (BTV8 NET2006/04 (GenBank Accession number JX680455), BTV1 RSArrrr/01 (JX680465),
150 BTV-2IT(L) (JN255870)), or synthesised commercially (Genscript), ((BTV-2RSA(WT)
151 (JN255930), BTV-9IT(L) (JN255910), BTV1SASEG9 (D10905), BTV25 (EU839845), BTV26
152 (JN255161)) and cloned into the pCI mammalian expression vector (Promega). BTV10
153 segment 8 (NC006007) was synthesized commercially (Genscript) and cloned into pCI. All
154 plasmids used in this study were sequenced before use. Plasmid pCMV-luc was obtained by
155 inserting the firefly luciferase (FLuc) open reading frame into pCDNA3.1 (Invitrogen) as
156 previously described (54). pRL-CMV (Promega) is similar to pCMV-luc but contains an intron
157 before the Renilla luciferase gene. p125Luc expresses FLuc under the control of the IFN-β
158 promoter (55). pISRE-Luc (Promega) contains five copies of the ISRE-binding sequence (56)
159 located upstream of the TATA like promoter region from the herpes simplex virus thymidine
160 kinase (HSV-TK) promoter.
161 Antisera used in this study included polyclonal rabbit antisera raised against the BTV NS4
162 and NS2 proteins as previously described (8). Antibodies against B23 (Sigma), α-tubulin
163 (Sigma), IRF-3 (clone FL-425, Santa Cruz) and NF-κB p65 (clone D14E12, cell signalling) were
164 obtained commercially.
165 **In vitro RNA transcription.** Capped and polyadenylated RNA for use in luciferase assays was
166 generated using the mMESSAGE mMACHINE® T7 Ultra Kit as recommended by the
167 manufacturers (Ambion, Life Technologies), using linearised pCMV-luc or pRL-CMV as a
168 template.
169 An RNA control (named EU-Luc-pA) for use in RNAseq and qRT-PCR experiments was
170 obtained as follows. Linearized pCMV-luc was transcribed using the Megascript® T7 kit
171 (Ambion, Life Technologies) according to the manufacturer's instructions, with the

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exception that the transcription reaction mix was supplemented with an analogue of uridine (5-Ethynyl Uridine [EU], Invitrogen, Life Technologies). The RNA was then polyadenylated following the polyA tailing procedure of the mMESSAGE mMACHINE® T7 Ultra Kit. All *in vitro* transcribed RNA was recovered using the RNeasy Mini Kit (Qiagen).

Labelling and extraction of nascent RNA for transcriptomic analyses. A549 cells were seeded in 6-well plates, incubated at 37°C for 24h and mock-infected or infected with BTV8wt or BTV8ΔNS4 at a MOI of 4. At 8, 12 and 16h post-infection (pi), nascent RNA was labelled with 0.4 mM EU for 90 min. Cells were lysed with 1 ml of TRIzol® spiked with 1 ng of EU-Luc-pA. Total RNA was then extracted using the TRIzol method and further purified using the RNeasy mini spin columns (Qiagen), including an on-column DNase I digestion step (Qiagen) according to the manufacturer's protocol.

RNAseq. 4.5 µg of total RNA was enriched by selectively depleting rRNA using the RiboMinus™ Eukaryote Kit v2 (Ambion, Life Technologies). The EU-labelled RNA was specifically linked to an azide-modified biotin by a chemical reaction and then captured on streptavidin magnetic beads using the Click-iT® Nascent RNA Capture Kit (Molecular probes, Life Technologies). Biotin-labelled RNA (attached to the magnetic beads) was fragmented and subsequently used to construct libraries using the Ion Total RNAseq v2 Kit (Life Technologies) as described by the manufacturer. Amplified libraries were size-selected using the E-Gel size select system (Life Technologies) and assessed using a Tapestation (Agilent). Libraries were quantified using the Qubit HS dsDNA assay (Life Technologies) and sequenced using the Ion Proton sequencer (Life Technologies). The sequence reads were processed according to the Tuxedo pipeline (57). Read quality was first assessed using FastQC (58). Tophat2 and Bowtie2 were used to map short reads against the *Homo Sapiens* genome (UCSC hg19) and a list of differentially expressed genes was generated using CuffDiff2

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(genes with Benjamini Hochberg *P*-value ≤ 0.05 were considered significant) (59, 60). Canonical pathway analysis was performed using the Ingenuity Pathway Analysis (IPA) software (Qiagen) by submitting the differentially expressed (DE) gene datasets (Tables S1-3) and using the default parameters.

qRT-PCR For qRT-PCR analysis, mRNA was purified from the total RNA fraction using the Dynabeads® mRNA DIRECT™ Kit (Ambion, Life Technologies) according to the manufacturer's instructions, before capturing EU-labelled RNA as described above. RNA captured on the Streptavidin beads was used directly as a template for cDNA synthesis using the SuperScript® VILO™ cDNA synthesis Kit (Invitrogen, Life Technologies). qPCR was performed using the Brilliant III Ultra Fast QPCR Master Mix (Agilent) to detect a selection of ISGs, housekeeping genes and the spiked EU-Luc-pA. Sequences of primers and probes are available upon request. Samples were run on an Mx3005P (Stratagene) PCR machine and analyzed using the MxPro software (Stratagene). Mock-infected cells were used as a calibrator against which the infected cells were compared. The EU-Luc-pA RNA was used as an exogenous control to normalise the results.

Interferon protection assays. Interferon protection assays were performed as previously described (46). Briefly, OvEC cells were first seeded in 12-well plates and incubated for 2 days. Cells were then mock-infected or infected with the indicated virus at a MOI of 4 and the medium was collected 16h post-infection. Cell culture supernatants were treated with UV light to inactivate any virus. CPT-Tert cells were incubated with two-fold dilutions of the medium for 24h, before infecting them with EMCV for 72h. The level of IFN (expressed as "international units", IU) was calculated by monitoring wells that were protected from cell death induced by EMCV and comparing them to known amounts of universal IFN.

Western blotting. Protein expression was assessed from total cell lysates by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting using the various antisera indicated above and as previously described (61). For quantitative western blotting, primary antibodies were detected using fluorescently labelled secondary antibodies (DyLight, Thermo Scientific) in a LI-COR Odyssey scanner. Bands were quantified with the Odyssey software (LI-COR Biosciences).

Metabolic radiolabeling. ovEC cells were mock-infected or infected with BTV8wt or BTV8ΔNS4 at a MOI of 4. At the time points indicated, cells were incubated at 37°C for 30 min in media lacking methionine. Nascent proteins were labelled for 2 h with ^{35}S methionine/cysteine (0.8 MBq/ml; PerkinElmer) after which they were resuspended in sample buffer (60 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 2.5% β-mercaptoethanol, 0.01% bromophenol blue) and the proteins separated by SDS-PAGE as described above. Visualisation of labelled proteins was achieved by exposure to a Storm840 Phospho-imager (Molecular Dynamics) or to X-ray film. Band intensities were determined using ImageQuant software (Amersham).

Luciferase assays. CPT-Tert cells grown in 24-well plates were co-transfected using Lipofectamine 2000 (Invitrogen) with 100ng of pCMV-luc or pRL-CMV along with 100 to 400 ng of the indicated pCI plasmids expressing NS4. A similar procedure has been performed to co-transfect 200 ng of *in vitro* transcribed RNA expressing firefly luciferase (see above) and the appropriate expression plasmids as indicated (100 to 400 ng). When using p125Luc (50 ng) and pISRE-luc (400 ng), 293T cells were either infected with DI-rich preparations of SeV or treated with 200 U/ml of Universal Interferon (UIFN, PBL InterferonSource) 4h post transfection, before incubating for a further for 18h.

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242 The total amount of plasmid DNA transfected used was 500ng for all experiments, using the
243 empty pCI plasmid to balance each transfection. Luciferase activity was detected 22h post-
244 transfection using the Luciferase or Dual Luciferase Assay System (Promega) as described by
245 the manufacturer. The percentage of luciferase was determined by assigning the luciferase
246 activity or luminescence (expressed as relative light unit, RLU) detected in control cells (i.e.
247 transfected with pCMV-luc and an empty pCI plasmid only) as 100%. Constitutive promoters
248 as transfection controls could not be used as NS4 affects gene expression. However, each
249 experiment was repeated independently at least three times with each samples assessed in
250 in triplicate. For each experiment, at least two independent plasmid preparations were
251 used. Relative lights units for each sample were always at least 100 fold above background.
252 In addition, in some experiments, 5 ng of *in vitro* transcribed RNA encoding renilla luciferase
253 were used as additional internal control.
254 **Confocal microscopy.** Experiments were performed using CPT-Tert or A549 cells cultured in
255 two-well glass chamber slides (Lab-Tek, Nalge Nunc International). Cells were either
256 transfected with the appropriate plasmids or infected with the indicated viruses (MOI = 4)
257 for 16-22h. Cells were washed with PBS and fixed with 5% formaldehyde for 15 min. Fixed
258 cells were then processed as described previously (62) and incubated with the appropriate
259 antisera. Secondary antibodies were conjugated with either Alexa Fluor 488 or Alexa Fluor
260 594 (Invitrogen, Molecular Probes). Slides were mounted using Vectashield mounting
261 medium with DAPI (4',6-diamidino-2-phenylindole, Vector Laboratories). Slides were
262 analysed and images collected using a Leica TCS SP2 confocal microscope.
263
264

RESULTS

BTV8ΔNS4 is attenuated in experimentally infected sheep. In a previous study, we showed that a BTV8 NS4 deletion mutant (BTV8ΔNS4) is as virulent as BTV8 wt in experimental mouse models of infection (8). Here we wanted to determine whether NS4 influenced virulence in sheep, the natural host of BTV infection. Therefore, we experimentally infected sheep with BTV8wt or BTV8ΔNS4. Sheep infected with BTV8wt showed an elevation of body temperature from day 6 to day 8 pi (Fig. 1, top panels). BTV RNA was detectable at day 4 pi, reached a plateau from day 6 to 10 pi and was followed by a slow decrease. Viremia remained detectable until the end of the experiment. In contrast, animals infected by BTV8ΔNS4 did not develop pyrexia and displayed a delayed onset and lower levels of viremia. BTV RNA was below the detection levels in 4 of the 5 inoculated sheep at 4 weeks pi when the experiment was stopped (Fig. 1, middle panels). Between days 4 and 28 pi, the average levels of BTV RNA were between 10^2 and 10^5 fold higher in sheep infected with BTV8wt compared to those infected with BTV8ΔNS4. As expected, all BTV infected animals developed neutralizing antibodies from day 7 pi (Fig. 1, lower panels). Altogether, these data show that BTV8ΔNS4 is attenuated in sheep and suggest that NS4 is a virulence factor *in vivo*.

Replication kinetics of BTV8wt and BTV8ΔNS4 in primary ovine endothelial cells and human A549 cells. We showed previously that BTV8ΔNS4 replicates as efficiently as BTV8wt in immortalized cell lines, such as hamster BSR and sheep CPT-Tert cells (8) but these cell lines do not possess an intact IFN response to viral infections (44, 45). Hence, we compared the replication kinetics of BTV8wt and BTV8ΔNS4 in primary ovine endothelial cells (ovEC). BTV8wt reached titers approximately 20 fold higher than BTV8ΔNS4 at 72h pi (Fig. 2A). Similarly, BTV8wt reached titers nearly 60 fold higher than BTV8ΔNS4 in the IFN-competent

human cell line A549 (Fig. 2A). These data demonstrate that the presence of NS4 confers a replication advantage to BTV8 in cells that are capable of mounting an antiviral response.

NS4 inhibits IFN release, but not virus sensing, in infected cells. Given the data obtained above and the previously published observation that NS4 confers a replication advantage to BTV in cells pre-treated with IFN (8), we next sought to determine whether NS4 impacted the level of IFN released into the supernatant of infected cells. Primary ovEC were infected at a MOI of 4 with either BTV8wt or BTV8ΔNS4. 16 h pi the level of IFN in cell culture media was measured using an IFN protection assay. BTV8ΔNS4 infected cells had, on average, 13 times more IFN ($p<0.001$) in their supernatants than BTV8wt infected cells (Fig. 2B).

These results could potentially be explained by NS4 affecting either the sensing of viral infection, or transcription and/or the synthesis/secretion of IFN into the supernatant. The first step in IFN-β expression is the recognition of pathogen-associated molecular patterns (PAMPs) by various host pattern recognition receptors, which results in the translocation of the transcription factors IRF-3 and NFκB into the nucleus (37). In order to determine whether NS4 impacted supernatant levels of IFN by interfering with this process, we infected A549 cells at a MOI of 4 for 16h and assessed the nuclear translocation of IRF-3 and NFκB by confocal microscopy. The cellular localization of IRF-3 and NFκB in mock-infected cells was exclusively cytoplasmic (Fig. 3) while stimulation with TNFα (tumor necrosis factor α) used as positive control resulted in nuclear translocation of these proteins in essentially 100% of the cells (not shown). In contrast, between 25 and 35% of cells infected by BTV8wt showed translocation of IRF-3 and NFκB into the nucleus. BTV8ΔNS4 was found to induce similar levels of translocation as BTV8wt, suggesting that NS4 does not prevent either PAMP recognition by the host cells or translocation of IRF-3 and NFκB. NS2 immunolabelling confirmed similar levels of infection in cells infected with BTV8wt or BTV8ΔNS4 (Fig. 3).

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313 **NS4 downregulates IFN-β and interferon stimulated genes (ISGs) mRNA levels.** Next, we
314 carried out RNAseq analysis on IFN-competent A549 cells infected with either BTV8wt or
315 BTV8ΔNS4 in order to further characterize the activity of NS4. Mock-infected A549 cells
316 were used as negative controls. Libraries were prepared from nascent RNA metabolically
317 labelled with a uracil analogue for 90 minutes at 12h pi (Fig. 4A), and sequenced on an Ion
318 Proton sequencer (Life Technologies, Thermo Fisher). On average, 64.58 million reads per
319 sample were generated (Phred quality > 20). We found 2863 differentially expressed genes
320 in cells infected by BTV8wt compared to mock-infected cells, out of which 1055 were
321 downregulated and 1808 were upregulated (Fig. 4B). In comparison, fewer genes (n=2292)
322 were found to be differentially expressed in cells infected by BTV8ΔNS4 compared to mock-
323 infected cells, with 752 of them being downregulated and 1540 upregulated. The entire list
324 of differentially expressed genes in BTV8wt and BTV8ΔNS4 infected cells is presented in
325 Tables S1-S3. CH25H (cholesterol 25-hydroxylase, a known ISG) was found to be the most
326 upregulated gene in BTV8ΔNS4 infected cells. 117 genes were upregulated in BTV8ΔNS4-
327 infected cells compared to BTV8wt-infected cells (Table S3). Of these 117 genes, 102 were
328 either IFN genes or ISGs according to the Interferome database (63). IFN-β, IFN-λ1, IFN-λ2
329 and IFN-λ3 were among the 6 highest upregulated genes in BTV8ΔNS4 infected cells (87 to
330 136 fold higher in comparison to mock-infected cells) (Table 1). Interestingly, more genes
331 were strongly upregulated (fold change >32) in BTV8ΔNS4 (n=33) compared to BTV8wt
332 infected cells (n= 7). These genes included MX1 and -2, IFIT1, -2 and -3, OASL and other well
333 characterised ISGs. However, all of the 33 genes highly upregulated in BTV8ΔNS4-infected
334 cells were also upregulated (albeit at a lower level) in BTV8wt infected cells (Table 1). IFN-β
335 for example, was also upregulated (17 fold) in BTV8wt infected cells. No major differences in
336 the levels of expression in BTV8wt and BTV8ΔNS4 infected cells were observed in those

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genes with the highest levels of downregulation (Table 2). We validated the RNAseq analysis by qRT-PCR on selected genes found equally or differentially expressed in mock- and infected cells. Relative mRNA levels of IFNB1, IFIT1, B2M, ACTB, ANXA1 and TBP determined by RT-qPCR reflected the patterns of expression observed in the RNAseq analyses (Fig. 4C). The Ingenuity Pathway Analysis software (IPA; Qiagen) was used to compare the representation of canonical pathways comparisons between infected vs uninfected cells (Fig 5A-B), and cells infected with either BTV-8wt or BTV8ΔNS4 (Fig 5C). Most of the pathways were involved in the cellular immune response, cytokine signalling, inflammatory response, apoptosis and pathogen related signalling. Consistent with the RNAseq dataset, pathways relating to the innate immune response were particularly evident when comparing BTV8ΔNS4 with BTV8wt (Fig. 5C).

The availability of the transcriptome of cells infected by BTV8wt and BTV8ΔNS4 also provided additional information regarding the possible influence of NS4 on mRNA maturation. Comparable proportions of reads containing intron sequences were found in mock-infected cells or in cells infected with BTV8wt or BTV8ΔNS4 (data not shown). In addition, we also quantified how many reads finished with 8 or more adenines, assuming these reads as representative of polyadenylated mRNAs. Comparable percentages of polyA reads were obtained in the three conditions tested, suggesting that NS4 was not associated with a global defect in mRNA polyadenylation (data not shown).

NS4 modulates the activity of a wide range of promoters. We next assessed the ability of BTV-8 NS4 to reduce the activity of basal promoters, such as the CMV immediate early promoter, and promoters of genes involved in the host innate immune response (IFN-β and ISRE-containing promoters). 293T cells were co-transfected with a plasmid expressing either BTV NS4 or NS2, and a FLuc reporter plasmid driven by either the CMV promoter, the IFN-β

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promoter or a promoter containing ISRE elements (Fig. 5). 4h post-transfection, cells transfected with the IFN-β promoter were stimulated with Sendai virus while cells transfected with ISRE-containing promoter were stimulated with universal IFN (200 U/ml). NS4, unlike NS2, was able to reduce between 40 and 60% the activity of both CMV, IFN-β and ISRE promoters (Fig. 6).

To further investigate the ability of NS4 to block host gene expression, sheep cells were co-transfected with an expression plasmid for FLuc, under the control of the CMV immediate-early promoter along with a variety of expression plasmids expressing BTV NS4, or an empty plasmid (also containing a CMV promoter) as a control (Fig. 7). NS4 is well conserved among the BTV serotypes/strains identified to date (8). The only exception was a strain of BTV-1 (GenBank accession number D10905 submitted in 1992) and the more divergent BTV-25 and BTV-26 strains (EU839845 and JN255161) which showed only 77.9%, 76.6% and 75.3% identity to BTV-8 NS4, respectively (Fig. 7A). All of the NS4 proteins tested were able to reduce FLuc expression with the notable exception of the NS4 from the BTV/D10905 (Fig. 7B). Interestingly, the NS4 protein of BTV-1 D10905 displayed a different mobility from other NS4 proteins by SDS-PAGE that could be explained by the presence of considerable differences in its basic residues in the N-terminus. In addition, using BTV-8 NS4 we showed that gene expression inhibition by NS4 was dose dependent (Fig. 7C).

In a previous study, we showed that NS4 localizes in the nucleoli of infected or transfected cells (8). Interestingly, BTV-1/D10905 NS4 was the only variant which failed to localise to the nucleoli of sheep CPT-Tert cells (Fig. 7D), suggesting that nucleolar localization may be critical for the activity of this non-structural protein.

NS4 is not the only viral protein involved in host cell protein shutoff. It is well established that BTV induces protein synthesis shutoff in infected cells (64-66). Conceivably, a reduced

level of IFN (both mRNA and proteins) observed in cells infected with BTV8wt could reflect the general virus-induced shut off of protein synthesis. In order to test this hypothesis, we metabolically radiolabelled nascent proteins in ovEC cells mock-infected, or infected with either BTV8wt or BTV8ΔNS4. As expected, we observed decreased levels of ^{35}S-labelled methionine/cysteine proteins in BTV8wt-infected cells compared to mock infected cells (particularly evident at 18 and 26h pi), confirming previously published data (Fig. 8) (65, 66). Protein synthesis shutoff was also evident in BTV8ΔNS4 infected cells, although at somewhat reduced levels compared to BTV8wt. In order to quantify the reduction in protein synthesis during viral infection we used phosphoimaging and measured the signal intensity of a prominent band present in all samples (Fig. 8A, black arrow). The signal intensity of actin in BTV8wt-infected cells relative to mock-infected cells (taken as 100%) reduced progressively to 69% at 10h pi, 37% at 18h pi and 2% at 24h pi, by which point cytopathic effect was apparent. On the other hand, the signal intensity of actin was 82%, 56% and 12%. Hence, these data suggest that host protein shutoff induced by BTV occurs largely independently of NS4, although the latter may contribute to this phenomenon.

BTV-8 NS4 does not influence mRNA splicing nor translation. We also assessed the impact of NS4 on RNA transcript splicing. CPT-Tert cells were transfected with the pRL-CMV vector, which is driven by the CMV immediate early promoter and contains the RLuc gene downstream of an intron. In this assay, NS4 retained the ability to inhibit the expression of the reporter gene in a dose-dependent manner (Fig. 8B), indicating that NS4 does not affect mRNA splicing and confirming the data obtained by RNAseq described above. Cells were also co-transfected with *in vitro* transcribed RNA encoding FLuc and an expression plasmid for the BTV8 NS4, in order to determine whether NS4-induced inhibition of protein expression could also occur at translational level (Fig. 8C). Increasing levels of NS4 did not

409 interfere with the level of FLuc activity driven by RNA, as opposed to the activity driven by
410 plasmid DNA used as control, suggesting that the inhibition mediated by NS4 is happening
411 most likely at transcriptional and not at the translational level.
412

DISCUSSION

In this study we showed that NS4 is an IFN-antagonist and a virulence factor for BTV. BTV NS4 deletion mutants replicate as efficiently as wild type viruses in cell lines that lack a competent innate immune system and are lethal to IFNAR$^{-/-}$ mice (8). In contrast, here we demonstrated that NS4 facilitates BTV replication *in vitro* in IFN competent cells, and *in vivo* in sheep, the animal species most affected by bluetongue.

BTV induces a type 1 IFN response *in vivo* and *in vitro* (38-42). Our data strongly suggest that NS4 is required by the virus to help overcome the cellular innate antiviral responses. Reporter assays revealed that NS4 can inhibit transcription from a variety of mammalian and viral promoters, whilst having little if any direct impact on mRNA editing or translation. However, NS4 does appear to influence the extent of the host IFN response as supernatants of primary sheep endothelial cells infected with BTV8ΔNS4 contained more IFN than supernatants collected from BTV8wt infected cells; a result which may explain the more efficient growth of BTVwt in IFN competent cells. These results were supported by RNAseq analyses of nascent RNA isolated from infected and mock-infected cells. Genes relating to the innate immune system were among the highest upregulated genes in BTV8ΔNS4 infected cells, even if some of them were also found upregulated in BTV8wt infected cells, suggesting that NS4 is not by itself sufficient to inhibit entirely the host IFN response. Most of the genes that were specifically upregulated in BTV8ΔNS4 infected cells, as compared to BTV8wt infected cells, were ISGs including well characterised antiviral factors.

Overall, the data obtained in this study indicate that NS4 exerts its effect upon the antiviral host response at a point upstream of RNA editing and translation. Interestingly, NS4 possesses features of a transcription factor of the bZip family, with a basic domain followed by a leucine zipper motif (67) and it has been suggested to have the ability to bind DNA

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437 using DNase protection assays (9). Our reporter assays, showing that NS4 inhibits
438 transcription from a variety of promoters, including IFN-β and an ISRE-containing promoter,
439 suggest that this protein may inhibit cellular transcription. In a previous study we showed
440 that NS4 displays a nucleolar localization in infected cells (8). Thus, a direct interaction of
441 NS4 with transcription factors and/or chromatin, resulting in the downregulation of cellular
442 transcription is a possible mechanism of action for this protein. In support of this
443 hypothesis, we found that the NS4 of a BTV-1 strain that did not show nucleolar localization
444 was not able to modulate gene expression in reporter assays. It is also possible that NS4
445 inhibits IFN-β activation before transcription. Nuclear translocation of IRF-3 and NFκB
446 appeared to occur at similar levels in both BTVwt and BTVΔNS4 infected cells, suggesting
447 that NS4 does not prevent PAMP recognition by the host cells. However, it is possible that
448 IRF-3 or NF-kB could be inhibited by NS4 in the nucleus. It is important to note that, in a
449 previous study, we showed that NS4 confers a replication advantage to BTV in cells pre-
450 treated with type I IFN, highlighting its ability to counteract the innate immune response
451 after IFN activation. Hence, it is less that NS4 blocks PAMP recognition and subsequent
452 signalling. More studies will be necessary to fully dissect how the NS4 counteracts the host
453 innate immune system. Obviously NS4, like other non-structural proteins of different
454 viruses, might have different functions and antagonise the IFN response via multiple
455 mechanisms. For example, the NS1 protein of Influenza A viruses inhibits IFN production by
456 blocking IRF3 and NF-κB activation, and mRNA maturation (68, 69).
457 It has been known for at least three decades that BTV induces host protein synthesis
458 shutdown (22, 64, 65). Recent studies suggest that BTV NS1 favours viral RNA translation
459 and therefore competes with cellular protein synthesis (22). A key difference between
460 mammalian and BTV transcripts is the lack of a polyA tail. The NSP3 protein of Rotaviruses

(also members of the *Reoviridae*) acts in a similar fashion and outcompetes the polyA binding protein of cells, thus biasing translation of viral transcripts (70, 71). It remains possible that a similar mechanism may exist for BTV. Our data, obtained in metabolically labelled cells, confirm that BTV induces host protein synthesis shutdown and also establishes that this occurs largely independently of NS4. Hence, other BTV proteins might also play a similar role in the shutoff of host cell protein synthesis or function in different ways as IFN antagonists. For example, NS3 is believed to modulate IFN induction downstream of RIG-I and upstream of IKKε activation. Therefore, NS3 and NS4 may potentially act synergistically to counteract the innate immune system (28). In addition, it has also been shown that BTV inhibits the IFN signalling pathway by downregulating key components of the JAK/STAT pathway: JAK1 and TYK2 (43). The downregulation of JAK1/TYK2 may be the result of a specific interaction with a BTV protein and/or due to the general host protein synthesis shutdown induced by this virus. We did not find either of these two genes to be differentially expressed in BTV8wt or BTV8ΔNS4 infected cells.

In this study, we showed that BTV8ΔNS4 infected sheep display lower levels of viremia, lasting for shorter periods of time, compared to the viremia observed in animals infected with BTV8wt. The levels and duration of viremia in infected animals are essential factors for successful transmission between the mammalian hosts and the intermediate arthropod vectors. As mentioned above, BTV infection can result in a lethal hemorrhagic fever in some animals while in others the virus induces a mild febrile illness or even a clinically unapparent infection. The data obtained in this study provide evidence that viral proteins modulating the host innate immune response play a significant role in viral pathogenesis. This study lays the foundations for a deeper understanding of the mechanisms by which BTV antagonises the IFN system, in turn helping us to define the molecular determinants of BTV virulence.

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Acknowledgements

This study was supported by a programme grant from the Wellcome Trust. The authors are grateful to Meredith Stewart for useful suggestions.

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Table 1. Top 33 upregulated genes in BTV infected compared to mock-infected A549 cells.

	BTV8ΔNS4 vs Mock		BTV8wt vs Mock	
Gene	**Rank***	**Fold change**	**Rank**	**Fold change**
CH25H	U1	142.3	U18	23.6
IFNB1	U2	136.4	U41	17.5
IFNL1	U3	105.6	U11	28.8
IFNL2	U4	91.0	U68	14.1
MX1	U5	88.6	U38	17.8
IFNL3	U6	87.7	U98	12.0
OASL	U7	87.6	U13	28.3
IFIT2	U8	84.7	U14	25.9
RSAD2	U9	78.7	U50	16.3
IFIT1	U10	75.9	U36	18.2
CXCL11	U11	71.6	U24	21.5
IFIT3	U12	71.0	U40	17.6
CMPK2	U13	64.1	U112	11.2
IFI44	U14	63.4	U25	21.4
MX2	U15	61.8	U76	13.6
CCR4	U16	56.3	U17	24.0
IFI27	U17	49.7	U170	8.9
DDX58	U18	48.4	U115	11.0
OAS2	U19	46.1	U5	33.6
IFIH1	U20	45.5	U22	21.9
CA1	U21	45.0	U9	30.9
ISG15	U22	43.7	U78	13.4
FOSB	U23	43.6	U3	35.0
EGR2	U24	42.5	U8	31.8
FOS	U25	39.9	U15	25.3
IL8	U26	39.3	U29	19.8
KLRC2	U27	36.8	U10	29.0
EGR1	U28	36.1	U6	32.9
SLC1A3	U29	35.9	U46	16.7
EGR4	U30	35.7	U59	14.9
BATF2	U31	33.5	U548	4.3
HERC5	U32	32.4	U69	14.0
IFI6	U33	32.3	U303	6.2

*Rank of upregulated genes. The associated number (e.g. U1, U2 etc) refers to the rank of the specified gene. For example, CH25H is the gene found to be the most upregulated in BTV8ΔNS4 infected cells compared to mock-infected cells.

Table 2. Top 20 downregulated genes in BTV infected compared to mock-infected A549 cells.

	BTV8ΔNS4 vs Mock		BTV8wt vs Mock	
Gene	**Rank***	**Fold change**	**Rank**	**Fold change**
CLDND2	D1	0.13	D157	0.30
CPN1	D2	0.13	D24	0.20
DHRS4L1	D3	0.14	D425	0.38
EMID1	D4	0.14	NA**	1.0
LOC100506	D5	0.16	D3	0.15
HMOX1	D6	0.16	D4	0.15
KRT82	D7	0.16	D73	0.26
SHH	D8	0.17	D5	0.15
NUPR1	D9	0.18	D11	0.18
KRT4	D10	0.18	D142	0.30
SFRP4	D11	0.19	D9	0.17
NRTN	D12	0.19	NA	1.0
EMILIN1	D13	0.20	D18	0.20
LOC113230	D14	0.20	NA	1.0
LINC00086	D15	0.20	D329	0.36
OSGIN1	D16	0.21	D10	0.18
KLHDC7A	D17	0.21	D1	0.12
ATOH8	D18	0.22	D2	0.14
MIR210HG	D19	0.22	D106	0.28
KRTAP4-1	D20	0.22	D27	0.21

*Rank of down-regulated genes. The numbers D1, D2 etc. refers to the rank of the specified gene (the most down-regulated gene in a given condition being ranked first).

**NA= not applicable as these genes have not been found to be differentially expressed in the samples considered.

FIGURES

Figure 1. Experimental infection of sheep with BTV8wt and BTV8ΔNS4. Three groups of sheep (n=5 per group) were mock-infected (grey circle) or infected with either BTV8wt (black square) or BTV8ΔNS4 (black triangle). (Top panel) Body temperature (average per group) of experimentally infected sheep. Rectal temperature in sheep is normally between 38.3 and 39.9°C (indicated by plain black lines). (Middle panel) BTV RNA in blood samples of experimentally infected sheep. Viral RNA was detected by qRT-PCR targeting Seg-5, and values are expressed as log10 (copy number per µg of total RNA). (Bottom panel) Box and whisker plot representing neutralizing antibodies in experimentally infected sheep at 7, 14, 21 and 28 days post-infection as indicated in Materials and Methods. Grey stars (Mock versus BTV8wt) and black stars (BTV8wt versus BTV8ΔNS4). ** = $p<0.01$; *** = $p<0.001$ (two-way Anova, Bonferroni post-tests).

Figure 2. NS4 modulates IFN synthesis in infected cells. (A) *In vitro* replication kinetics of BTV8wt and BTV8ΔNS4 in primary ovine endothelial cells (ovEC) and human A549 cells. Cells were infected by BTV8wt (red) and BTV8ΔNS4 (blue) at a MOI of 0.01 and supernatants titrated at the indicated time points as described in Materials and Methods. Experiments were performed independently three times. Two-way ANOVA test, $p<0.01$. * = $p<0.05$; ** = $p<0.01$ (Bonferroni post-tests). (B) Upregulation of IFN-β synthesis in BTV8 infected cells. IFN protection assay. Primary ovine endothelial cells (ovEC) were mock-infected or infected with the indicated viruses (MOI=4). Supernatants were then collected at 16h pi, inactivated by UV treatment and the amount of IFN released in the supernatants determined in biological assays as described in Material and Methods. Bars in the figure represent

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734 standard deviation. One-way Anova p=0.002. *** = p<0.001 (Tukey's Multiple Comparison
735 Test).

736 **Figure 3. IRF-3 and NFκB nuclear translocation in BTV infected cells.** A549 cells were
737 infected at a MOI of 4 for 16h, and fixed before being processed for immunofluorescence
738 using antibodies against NS2, IRF-3 and NFκB with an Alexa Fluor 488 secondary antibody
739 (shown in green). Nuclei have been stained with DAPI (blue). Average values corresponding
740 to the percentage of translocation have been indicated for each experimental condition +/-
741 standard deviation. Scale bars correspond to 47.62 µm.

742 **Figure 4. RNAseq of BTV8wt-, BTV8ΔNS4- and mock-infected cells.** A. Schematic
743 representation of RNA sequencing workflow. A549 cells were mock-infected or infected
744 with BTV8wt or BTV8ΔNS4 at a MOI of 4. At 12h p.i., nascent RNA was metabolically labelled
745 with 0.4 mM of an analogue of uridine (5-ethynyl uridine, EU) for 90 m. Total RNA was
746 extracted and enriched by selectively depleting rRNA transcripts. The EU-labelled RNA were
747 chemically linked to an azide-modified biotin and then captured on streptavidin magnetic
748 beads. Biotin labelled RNA attached to the magnetic beads was used directly to construct a
749 library and subsequently sequenced using the Ion Proton sequencer. Sequence reads were
750 processed as described in Materials and Methods. B. Plots representing differentially
751 expressed (DE) genes according to their fold change values. Log2 fold change values >1 are
752 regarded as upregulated (q-value < 0.05), whereas <1 is regarded as statistically
753 downregulated (q-value < 0.05). C. Validation of RNAseq by qRT-PCR. mRNA levels of β-actin
754 (ACTB), Annexin A1 (ANXA1), TATA-binding protein (TBP), beta-2 microglobulin (B2M), IFN-β
755 (IFNB1) and interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) were
756 measured by qRT-PCR at 8 and 16h pi as described in Materials and Methods. * = p<0.05; **
757 = p< 0.01; *** = p<0.001 (One-way Anova, Tukey's Multiple Comparison Test).

758 **Figure 5. Cellular pathways of BTV8wt- and BTV8ΔNS4-infected cells.** A-C. Canonical
759 cellular pathways were analysed using Ingenuity Pathways Analysis. Only canonical
760 pathways with a p-value < $6.3x10^{5}$ are shown. Ratio corresponds to the number of genes
761 found DE in a given pathway divided by the total number of genes contained in that
762 pathway. A specific pathway is coloured in orange or blue depending on whether it has been
763 predicted to be activated (positive z-scores) or inhibited (negative z-scores), respectively.
764 Grey bars are pathways where no prediction can currently be made. PRR: Pattern
765 Recognition Receptors, AII: Antiviral Innate Immunity, DC: dendritic dell, MΦ: macrophages,
766 EC: endothelial cells, RA: rheumatoid arthritis, GC: gastric cells, NKC: natural killer cells, AR:
767 antiviral response, OSR: oxydative stress response, DM: diabetes mellitus. A. BTV8ΔNS4
768 versus mock-infected cells. B. BTV8wt versus mock-infected cells. C. BTV8ΔNS4- versus
769 BTV8wt-infected cells.

770 **Figure 6. Effects of NS4 on expression driven by various promoters.** CPT-Tert cells were co-
771 transfected with 100 ng of a DNA plasmid expressing either BTV10 NS2 (GenBank #
772 NC006007), or BTV8 NS4 and a DNA plasmid expressing FLuc under the control of either
773 CMV (50 ng), IFN-β (50 ng) or IFN-stimulated response element-containing (ISRE, 400 ng)
774 promoter. After transfection (4h), cells transfected with the IFN-β promoter were
775 stimulated with Sendai virus while cells transfected with ISRE-containing promoter were
776 stimulated with universal IFN. FLuc activity was assessed 22h post-transfection in a
777 luminometer. 5ng of capped and polyadenylated RNA made *in* vitro were also co-
778 transfected as a control of transfection efficiency. One-way Anova p=0.0002 (CMV),
779 p=0.0015 (IFN-β) and p=0.0006 (ISRE). * = p< 0.05; *** = p<0.001 (Dunnett's Multiple
780 Comparison Test).

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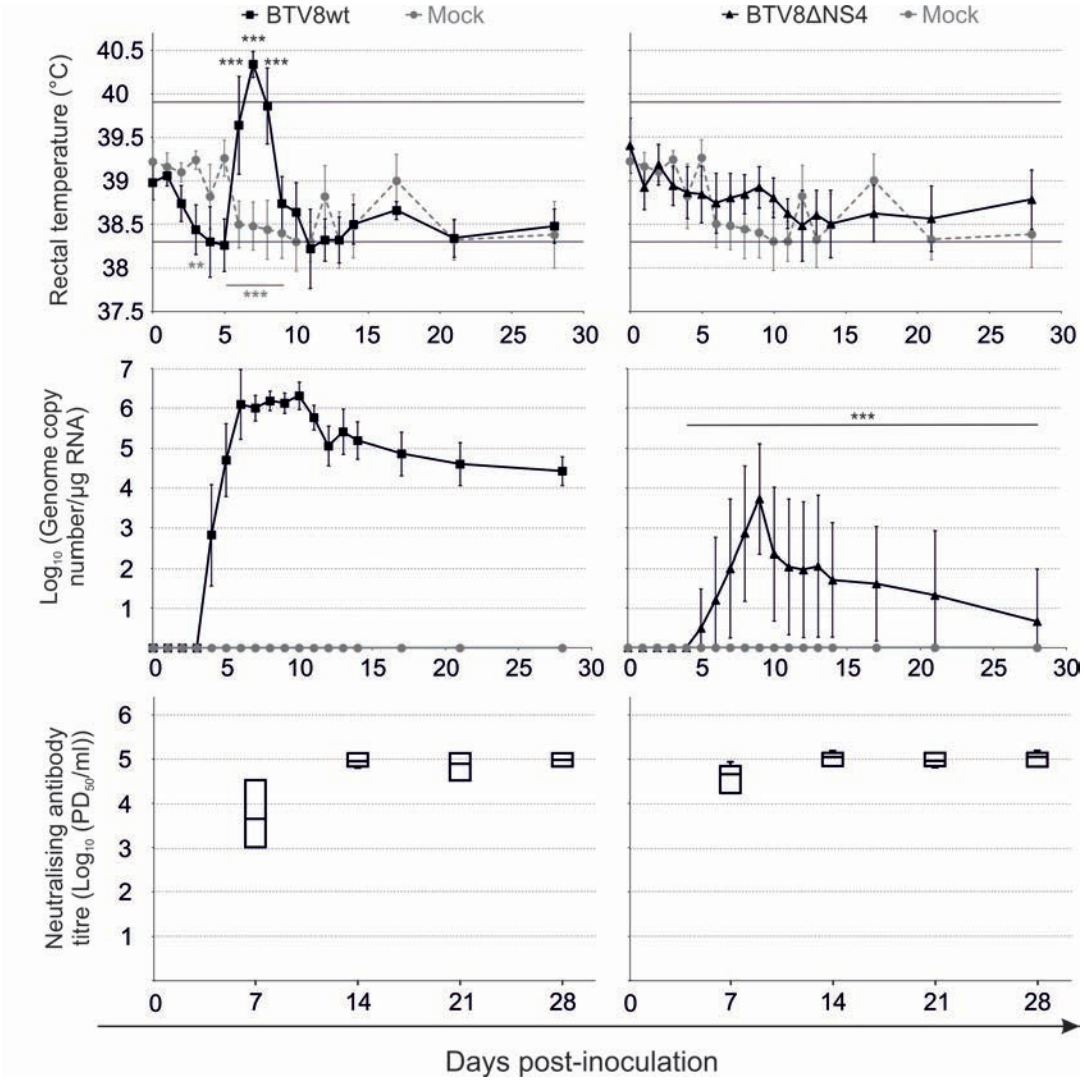
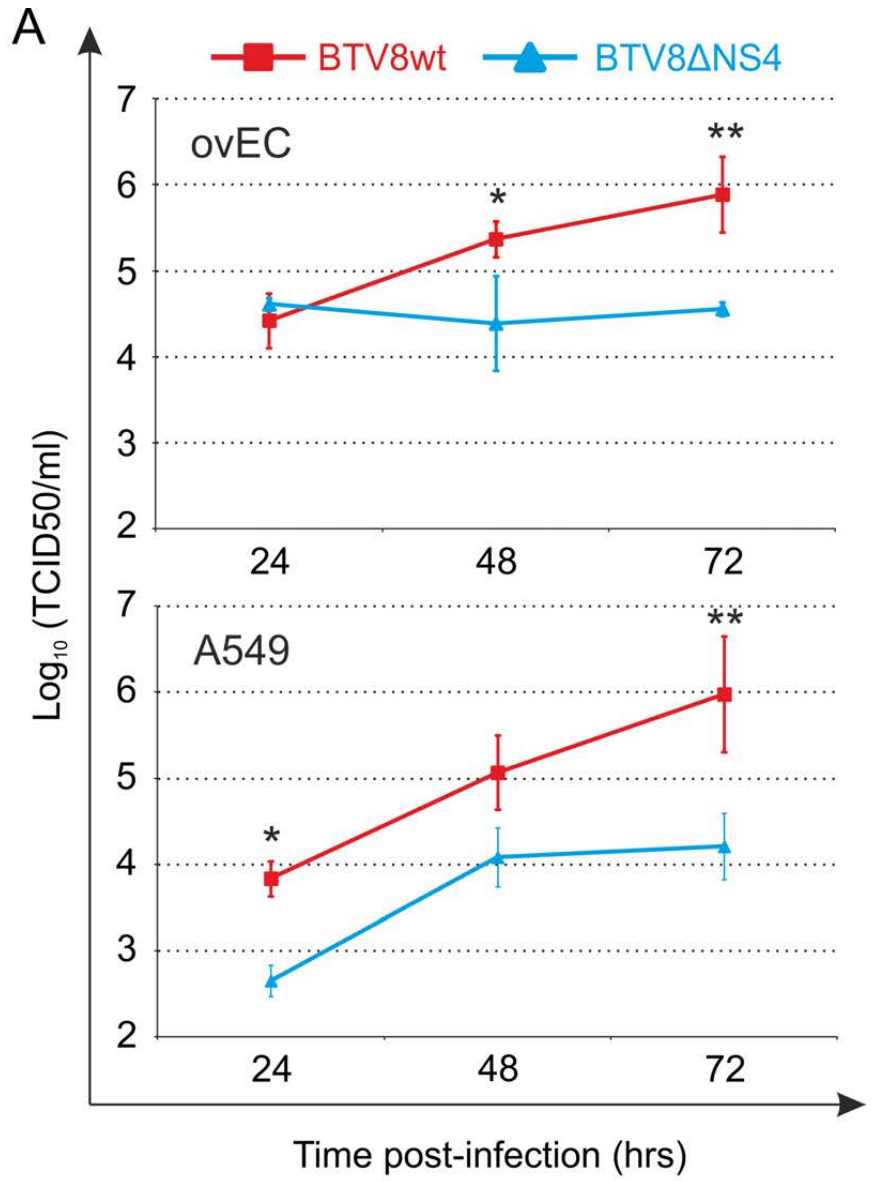
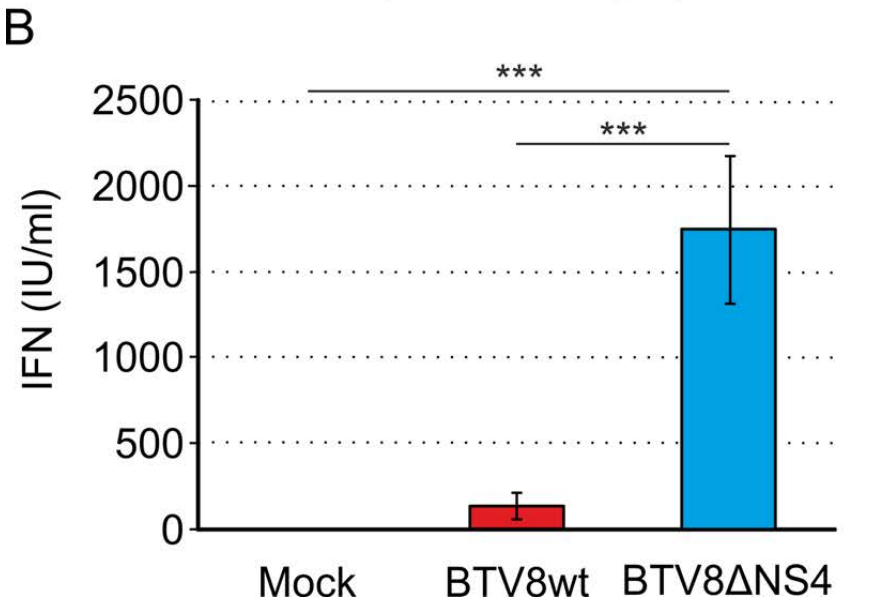
781 **Figure 7. NS4 from various BTV strains display inhibitory activity on gene expression.** A.
782 Multiple sequence alignment of representative NS4 sequences. Identical residues compared
783 to BTV8 (GenBank accession # JX680455) are shown as dots. Accession numbers refer to the
784 BTV genome segment 9 containing the NS4 open reading frame. B. CPT-Tert cells were co-
785 transfected with a plasmid expressing the indicated BTV NS4 protein (named according to
786 the accession number corresponding to the BTV segment 9) and a plasmid expressing FLuc
787 under the control of a CMV promoter. FLuc activity was assessed 22h post-transfection, and
788 in parallel by western blotting analysis of the same cell lysates using anti-NS4 or anti-
789 αtubulin antibodies. One-way Anova $p<0.0001$. *** = $p<0.001$ (Dunnett's Multiple
790 Comparison Test). C. CPT-Tert cells were co-transfected with increasing amounts of an
791 expression plasmid for the BTV8 NS4 and a plasmid expressing the firefly luciferase (FLuc)
792 under the control of the CMV immediate early promoter. FLuc activity was assessed 22h
793 post-transfection in a luminometer and in parallel by western blotting of the same cell
794 lysates using antibodies towards the NS4 or tubulin antibodies. One-way Anova $p<0.0001$.
795 ** = $p< 0.01$; *** = $p<0.001$ (Dunnett's Multiple Comparison Test). D. Confocal microscopy
796 of CPT-Tert cells transfected with pCI-NS4 plasmids. At 22h post-transfection, cells were
797 fixed and analysed by immunofluorescence using antibodies against the nucleolar marker
798 B23 (in red) and NS4 (in green), with the appropriate conjugated secondary antibodies as
799 described in Materials and Methods. Scale bars correspond to 33.21 µm.

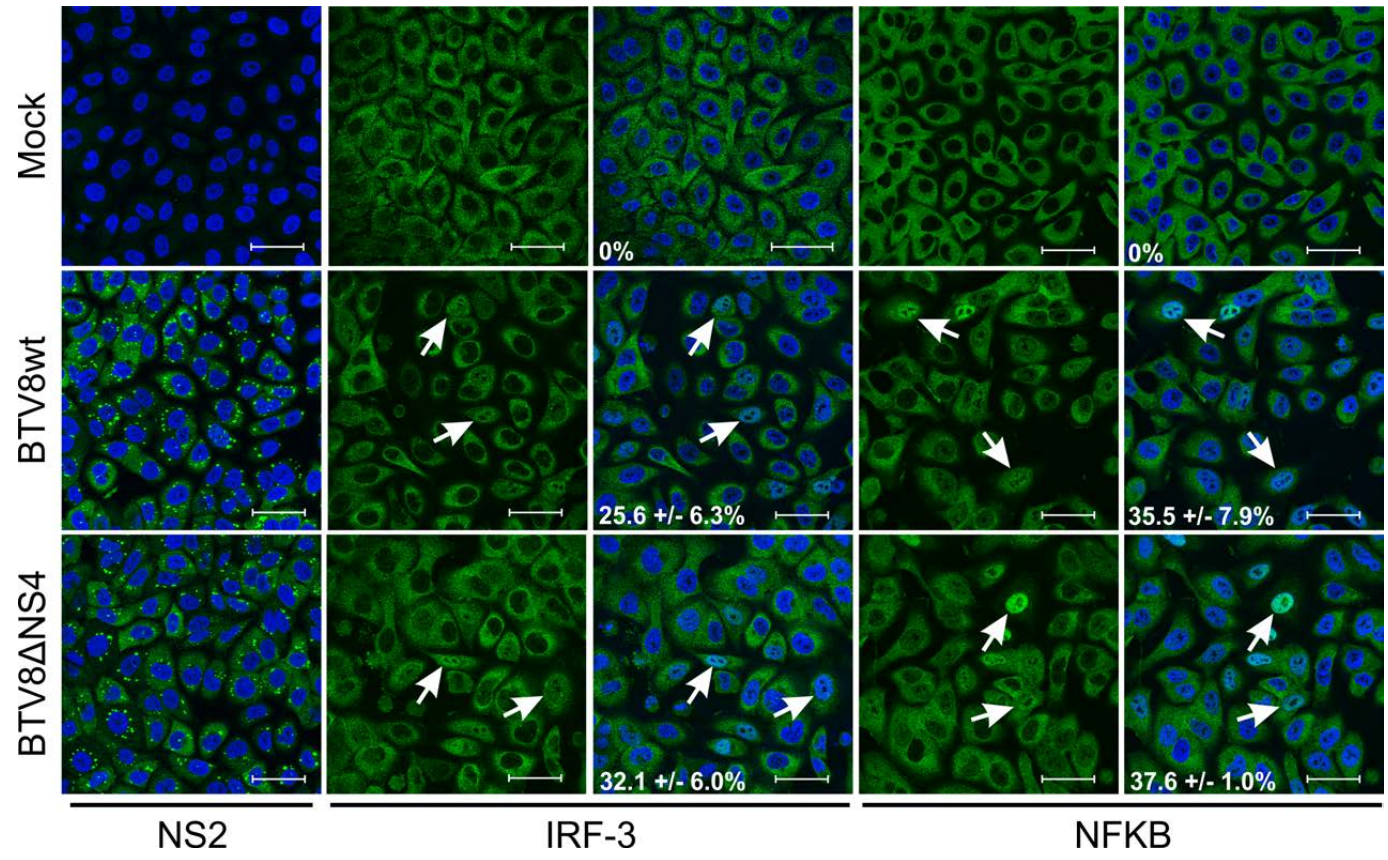
800 **Figure 8. NS4 expression does not affect significantly host cellular protein shutoff, mRNA**
801 **splicing or translation.** A. Primary ovEC were mock-infected or infected with BTV8wt and
802 BTV8ΔNS4 and nascent proteins were metabolically labelled with ^{35}S-methionine/cysteine
803 for 2 h at the time points indicated. Cell extracts were fractionated by SDS-PAGE and gels
804 were stained with Coomassie blue. Dried gels were analysed by phosphoimaging. Black

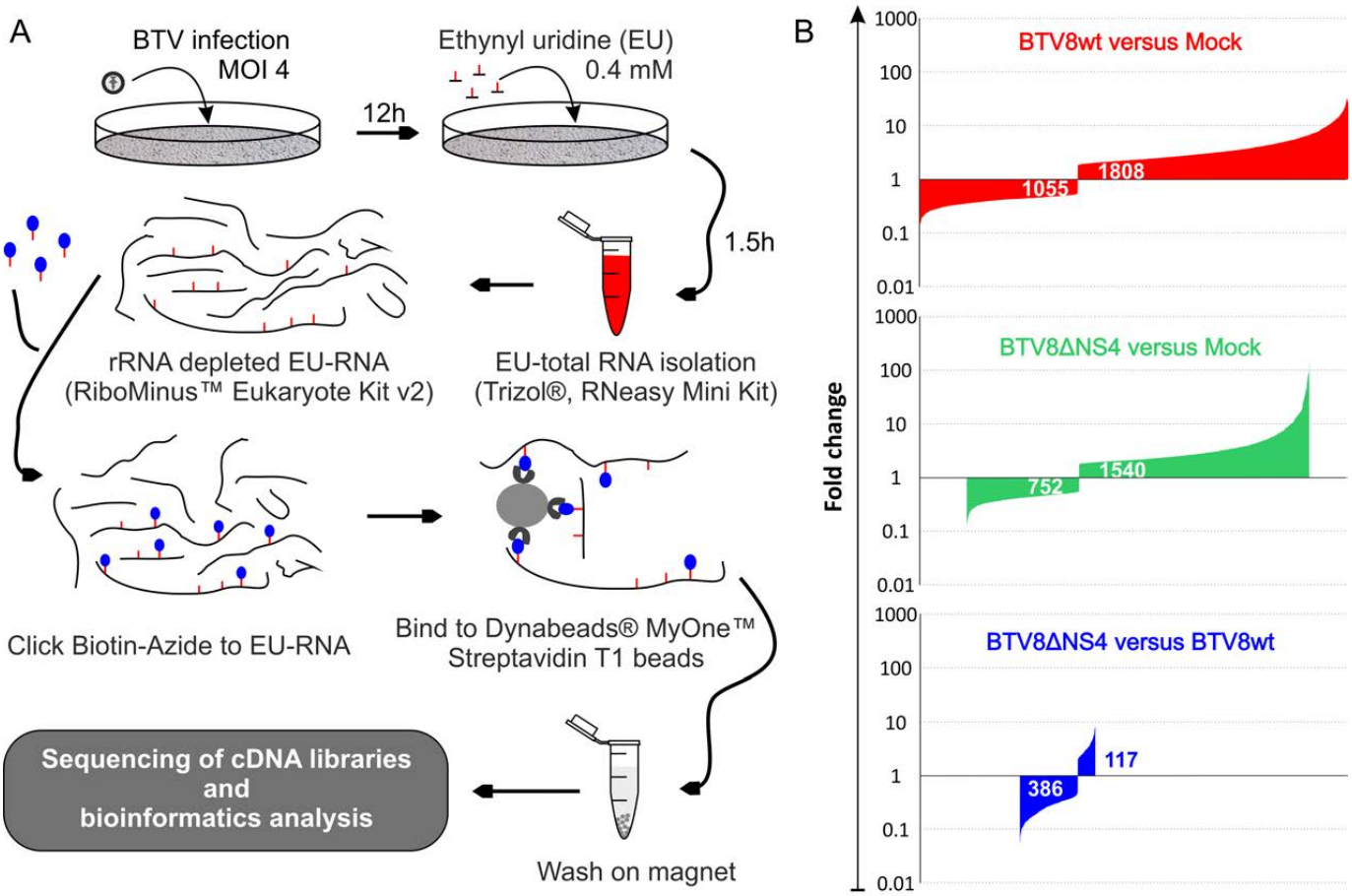
arrow indicates cellular actin. Signal intensity quantified using the ImageQuant software. Red arrows indicate BTV proteins. B. CPT-Tert cells were co-transfected with variable amounts of plasmids expressing BTV8 NS4 protein and either a plasmid expressing FLuc under the control of a CMV promoter or a plasmid expressing renilla luciferase (RLuc) downstream an intron and the CMV promoter. FLuc or RLuc expression was assessed 22h post-transfection. Cell lysates were analysed by western blotting using antibodies towards NS4. One-way Anova $p<0.0001$. ** = $p< 0.01$; *** = $p<0.001$ (Dunnett's Multiple Comparison Test). C. CPT-Tert cells were co-transfected with variable amount of a DNA plasmid expressing BTV8 NS4 and either a plasmid expressing the firefly luciferase (FLuc) under the control of a CMV promoter, or capped and polyadenylated RNA made *in vitro*. FLuc activity was assessed 22h post-transfection. Cell lysates were also analysed by western blotting using antibodies towards NS4. One-way Anova $p<0.0001$. * = $p< 0.05$; *** = $p<0.001$ (Dunnett's Multiple Comparison Test).

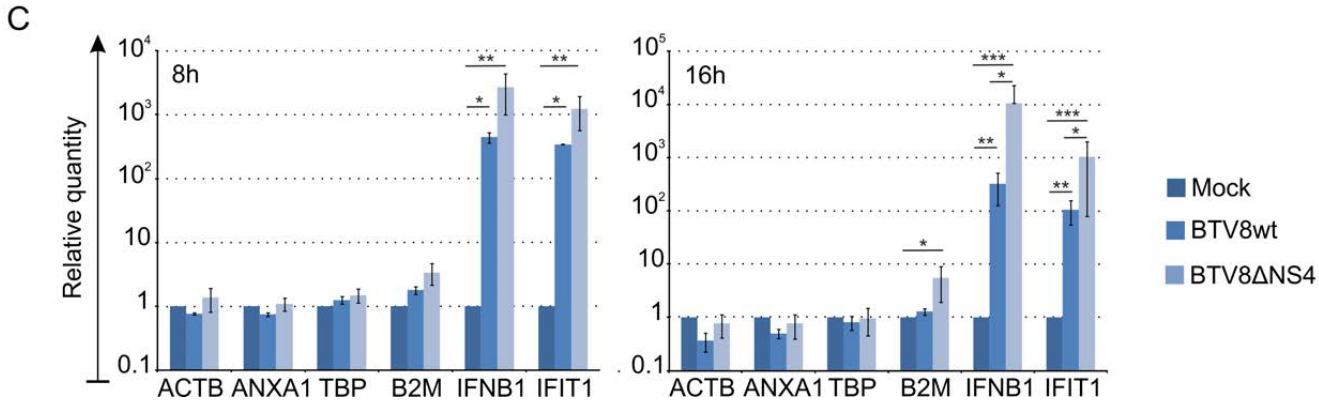


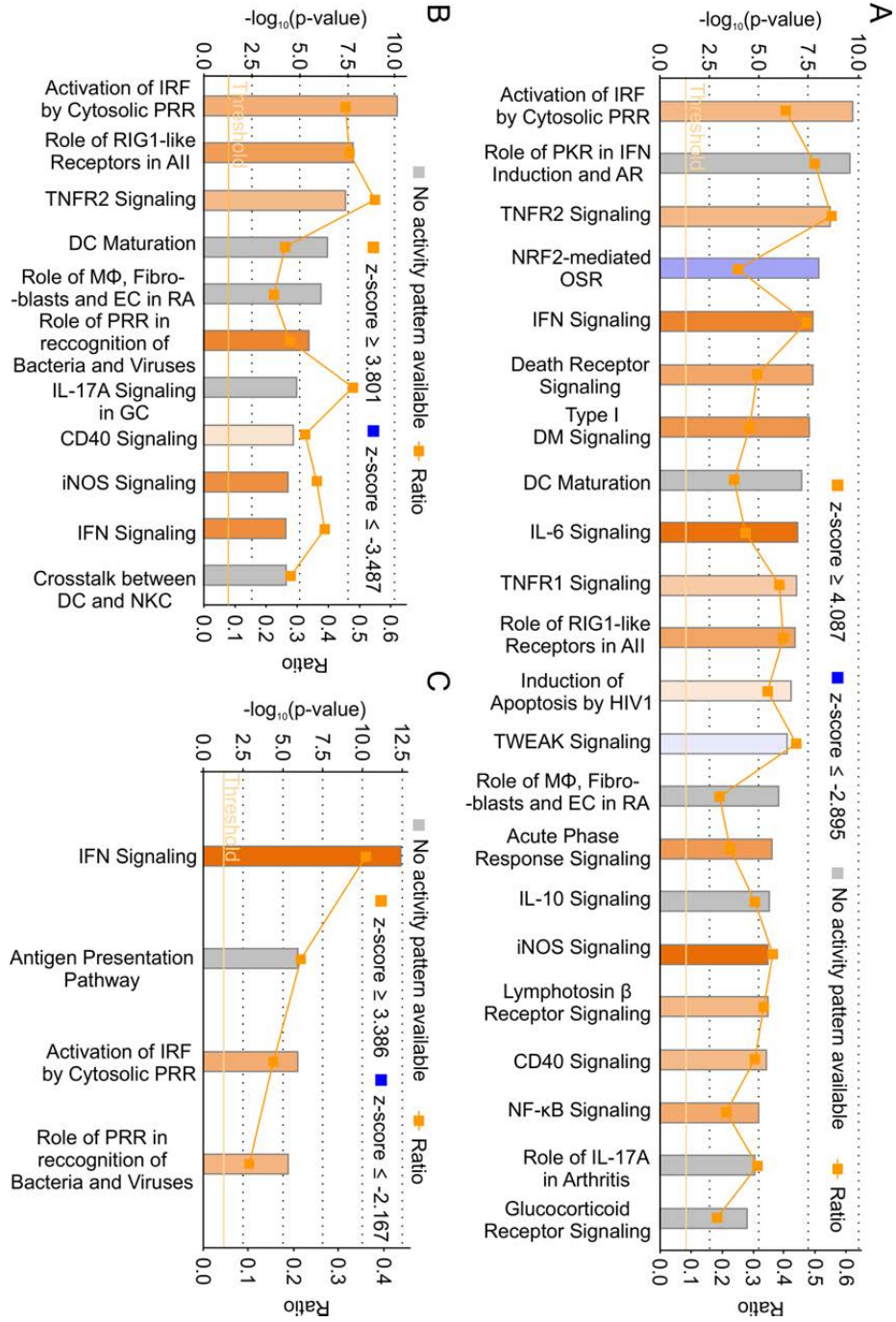








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Activation of IRF by Cytosolic PRR
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TNFR2 Signaling
NRF2-mediated OSR
IFN Signaling
Death Receptor Signaling
Type I DM Signaling
DC Maturation
IL-6 Signaling
TNFR1 Signaling
Role of RIG1-like Receptors in AII
Induction of Apoptosis by HIV1
TWEAK Signaling
Role of MΦ, Fibro--blasts and EC in RA
Acute Phase Response Signaling
IL-10 Signaling
iNOS Signaling
Lymphotosin β Receptor Signaling
CD40 Signaling
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Role of IL-17A in Arthritis
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TNFR2 Signaling
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Role of MΦ, Fibro--blasts and EC in RA
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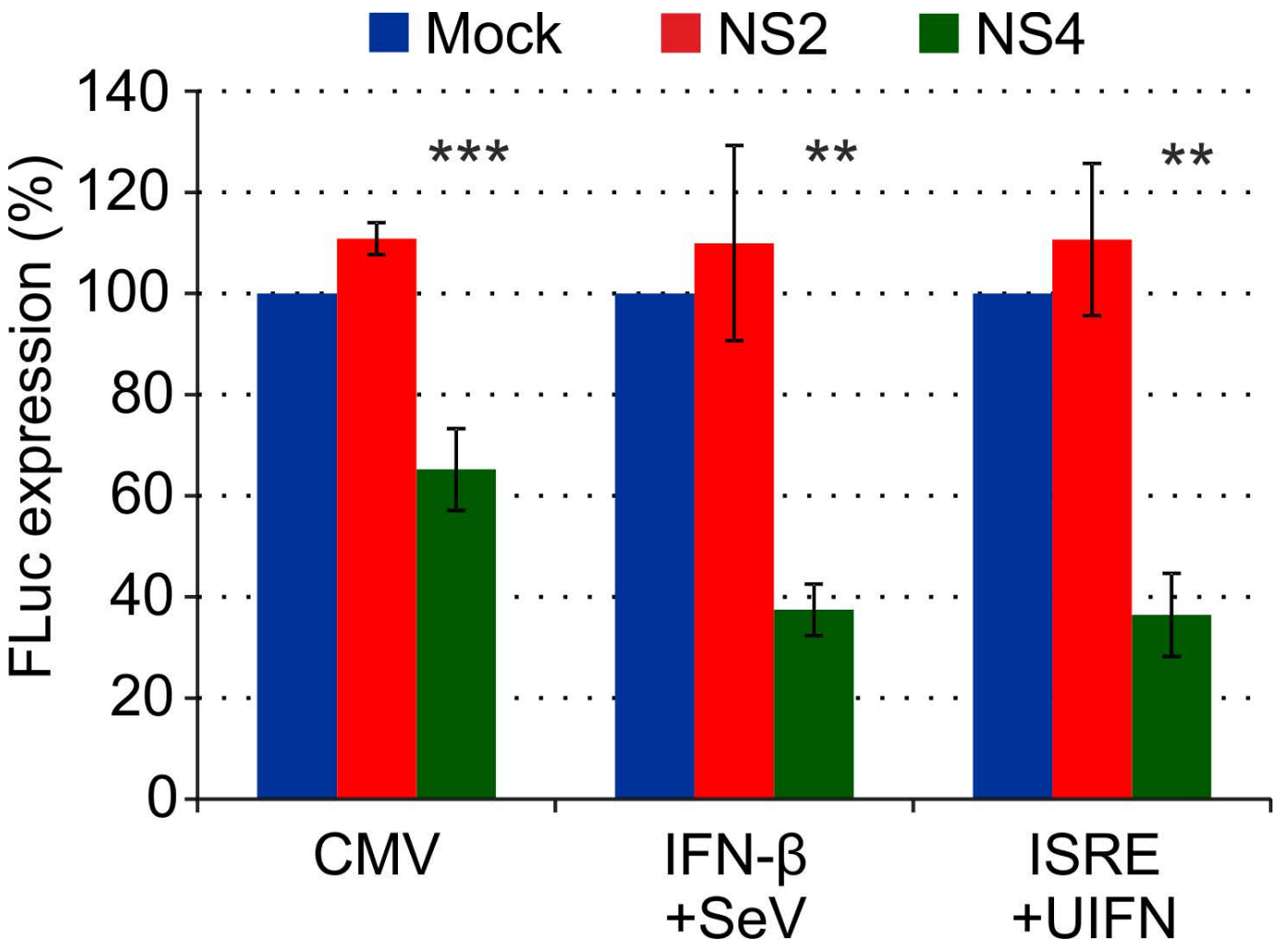
Ratio



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JN255870_BTV2       ..................................R.....................................-- 77
JN255930_BTV2       .....N.....................................................................WF 79
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D10905_BTV1         ...DAIGEQPEEREQP.....PDV....T.............................................-- 77
EU839845_BTV25      ...RQN...........K......M.............RE..E.Q...Q.......QD.I.L....M..M.Q.R...-- 77
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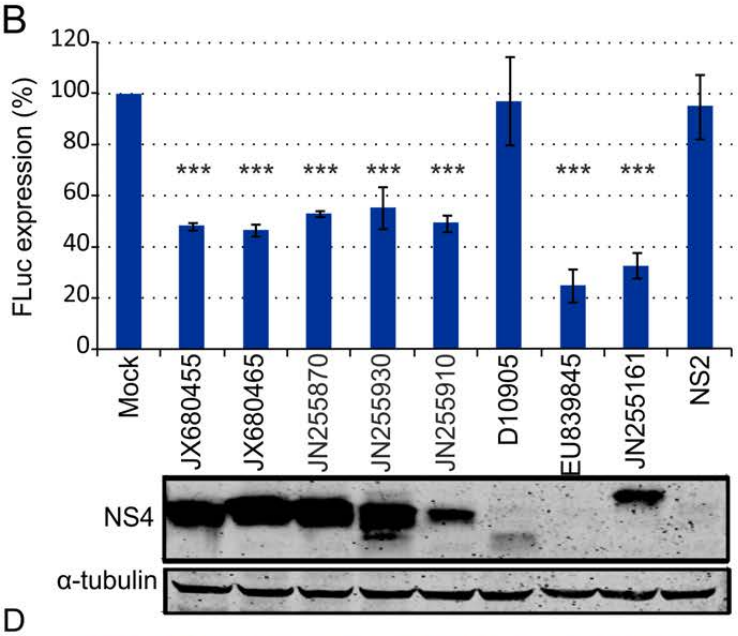

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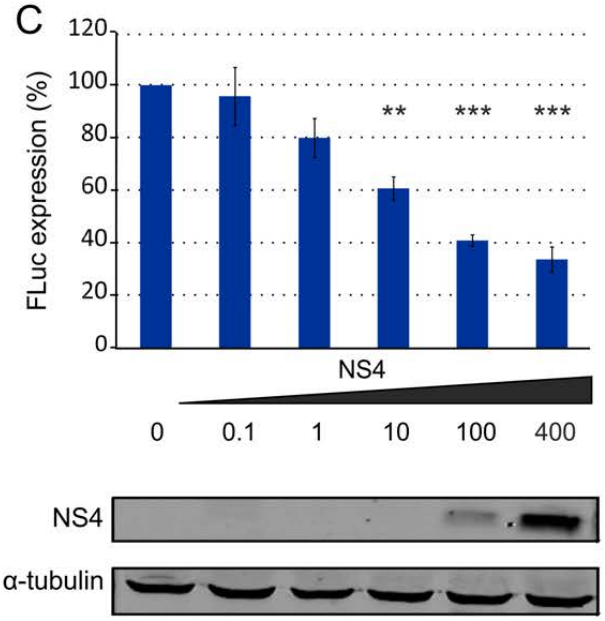

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